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Escape from the antiproliferative effect of transforming growth factor-β_1 in LLC-PK$_1$ renal epithelial cells

ROBERT J. ANDERSON, HEATHER T. SPONSEL, DAVID J. KROLL, STEVEN JACKSON, RUTH BRECKON, and JAMES P. HOEFFLER

Department of Medicine, Denver Veterans Affairs Medical Center, and Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, Colorado, USA

Escape from the antiproliferative effect of transforming growth factor-β_1 in LLC-PK$_1$ renal epithelial cells. Transforming growth factor-β_1 (TGF-β_1) usually inhibits proliferation of epithelial cells. We find that LLC-PK$_1$ renal tubular epithelial cells develop rapid *in vitro* resistance to the inhibitory effects of TGF-β_1 and subsequently proliferate in response to TGF-β_1. This unique response to TGF-β_1 is not observed in another renal tubular epithelial cell line (MDCK cells). The proliferative response to TGF-β_1 is additive to that produced by other growth factors. The proliferative response to TGF-β_1 occurs despite an effect of TGF-β_1 to suppress epidermal growth factor stimulated c-*myc* mRNA as determined by Northern analyses. These results suggest that LLC-PK$_1$ cells develop rapid resistance to TGF-β_1 inhibition of proliferation *in vitro* and that this resistance occurs despite continued suppression of c-*myc* mRNA.

Transforming growth factor-β_1 (TGF-β_1) is a 25 kD dimeric protein that belongs to a family of closely related peptides [1–3]. The TGF-βs exert numerous effects on growth and differentiation in multiple cell types [1, 2]. Most cells have receptors for TGF-βs and these peptides are felt to regulate cell function by acting as paracine and autocrine systems [1–3]. Initially, TGF-βs were found to stimulate anchorage-independent growth of fibroblasts in soft agar [4]. Later studies revealed that TGF-β_1 often inhibits the proliferation of numerous cell types [1]. The mechanism(s) whereby TGF-β exert(s) this antiproliferative effect remains unclear. However, several studies find that the growth inhibitory properties of TGF-β_1 occur in parallel with inhibition of transcription of the c-*myc* oncogene [5–15]. A role for the retinoblastoma tumor suppressor gene product (pRB) in mediating TGF-β_1 suppression of c-*myc* transcription has been demonstrated in some studies [10, 12, 16]. More recently, an effect of TGF-β_1 to prevent progression of the cell cycle beyond the G_1 phase has been attributed to inhibition of a cyclin-dependent kinase [17]. This inhibition correlated with diminished phosphorylation of pRB [17].

Recently, we investigated the effects of several putative renal epithelial cell growth regulators on signal transduction pathways in cultured renal cells [18]. To our surprise, we found that LLC-PK$_1$ pig kidney epithelial cells develop rapid *in vitro* resistance to the antiproliferative effect of TGF-β_1. The present studies were designed to characterize the response of LLC-PK$_1$ cells to prolonged *in vitro* exposure to TGF-β_1. Our results demonstrated that initially TGF-β_1 inhibits ^{3}H-thymidine uptake and proliferation in LLC-PK$_1$ cells. However, after 18 hours exposure, TGF-β_1 no longer inhibits and in fact significantly stimulates ^{3}H-thymidine uptake and proliferation. Several experiments were carried out to characterize the mechanism of this unique, biphasic response of LLC-PK$_1$ cells to TGF-β_1.

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Methods

Materials

Human recombinant TGF-β_1 and TGF-α were obtained from R and D Systems. Phorbol 12-myristate 13-acetate was obtained from Sigma. Human recombinant epidermal growth factor (EGF) and insulin-like growth factors 1 and 2 (ILGF-1 and ILGF-2 respectively) were obtained from UBI. Nitrocellulose membranes were obtained from Schleicher and Schuell.

Cell culture

LLC-PK$_1$ and MDCK cells were obtained from the American Type Culture Collection. LLC-PK$_1$ cells were grown in RPMI medium and MDCK cells in MEM medium supplemented with 2.0 mM glutamate. Both media were supplemented with 200 mg% sodium bicarbonate, 16 mM Hepes, 100 μ/ml penicillin, and 200 μg/ml streptomycin. Newborn calf serum (6%) was added to the media for the initial three to seven days of growth after which all cultures received serum-free media. Serum-free conditions were used for 48 hours prior to each experiment. The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell growth was monitored under an inverted microscope with phase optics (Nikon IM35) and typically reached confluence in five to seven days.

^{3}H-thymidine uptake and cell-counting studies

These studies were performed in 24-well panels in which cells were growth to approximately 70 to 80% confluence. After exposure to various effectors, media was replaced with fresh media containing [methyl-^{3}H]-thymidine (70 to 85 Ci mmol, Amersham, 5 μl/ml media) with or without 6% newborn calf

serum for four hours. Monolayers were then washed three times with cold PBS, three times with cold 5% TCA and cells solubilized in equal parts 0.25 N NaOH and 0.1% SDS. Radioactivity was determined by liquid scintillation counting. For each experiment, three to four wells were studied under each experimental condition and the three to four results meaned to obtain an N of one for that condition. Each experiment was typically repeated on three to five occasions. In all experiments, control cells were handled identically to experimental cells. When cell counts were done, cells were washed three times with PBS and removed from the dish with trypsin-EDTA (1:25 or 0.5 g trypsin and 0.2 g EDTA Na per liter), diluted in serum-free media and counted on a hemocytometer.

Northern blot analyses

For each experiment, 4 to 5 $\times$ 10^6 cells from a single 100 mm plate were used for each study condition. In all studies, poly(A)$^+$-RNA from cells was extracted using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, California, version 1.1). Approximately, 2 to 4 μg of poly(A)$^+$-RNA was electrophoresed through a 1.0% agarose gel with 2.2 M formaldehyde. RNA was transferred to nylon membrane overnight (Nytran, Schleicher and Schuell, Keene, New Hampshire, USA) by capillary action. RNA was fixed to the membrane by short-wave UV cross-linking (UV Stratalinker, Stratagene, La Jolla, California, USA). Prehybridization was performed at 42°C for two hours using a buffer composed of 5× SSPE (SSPE contains 149 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5× Denhardt's solution (1× contains 0.1 g Ficoll, 0.1 g polyvinylpyrrolidone, 0.1 g bovine serum albumin and H_2O to 500 ml), 0.1% sodium dodecyl sulfate (SDS), 100 to 200 μg/ml of sheared, denatured salmon sperm and 50% formamide (vol/vol). In more recent experiments, another prehybridization buffer was used containing 10× SSPE, 50× Denhardt's, 10.0% SDS, 200 to 300 μg/ml of sheared, denatured salmon sperm, 40% formamide (vol/vol), and 20% dextran sulfate (500,000 MS). Hybridization was conducted for 12 to 24 hours at 42°C using either a commercially available cDNA probe or a cDNA insert separated from its plasmid in low-melt agarose. The probes were labeled with about 5 μCi of [^{32}P]-dATP (3000 Ci/mol, ICN) using random primers. The cDNA probes used were a 2.0 kb fragment representing the chicken β-actin mRNA (Oncor) and a 2.3 kb Bam H1 fragment containing exons 1, 2 and 3 of the mouse c-*myc* gene (gift of Michael Cole). Hybridization was performed in prehybridization buffer containing 1 $\times$ 10^6 cpm/ml of [^{32}P]-labeled cDNA probes. After hybridization, membranes were washed twice for 15 minutes in 6× SSPE/0.1% SDS at room temperature and twice for 15 minutes in 1× SSPE/0.1% SDS at 37°C. The membranes were then autoradiographed with intensifying screens at −70°C for 24 to 96 hours. Blots were stripped with 50% formamide/6× SSPE at 65°C for 30 minutes followed by rinsing in 2× SSPE. After stripping, blots were subsequently rehybridized with other probes as described above. All experiments were repeated five times and representative autoradiographs are shown. A Bio-Rad model 620 densitometer using 1-D analyst 11 data analysis software (version 3.1) was used to obtain relative values for mRNA expression. For mRNA analyses, densitometric values for c-*myc* were divided by the corresponding value for β-actin. The values of these ratios from individual experiments were then averaged.

Western blot analyses

Whole cell lysates were made by removing cells from the culture dish with trypsin/EDTA. Cells were washed in PBS, then PBS with 5 mM $MgCl_2$ and then suspended in a hypotonic lysis buffer containing DNase I (100 μg/ml), RNase A (50 μg/ml), pepstatin (10 μg/ml), leupeptin (10 μg/ml), PMSF (1 mM), $MgCl_2$ (50 mM) and Tris 5.0 mM. The suspension was treated with 2.5% SDS and 100 mM DTT. Seventy-five micrograms of whole cell lysate were separated on denaturing SDS-polyacrylamide gels. The separated proteins were transferred to a nitrocellulose membrane in a Hoefer Scientific Transphor apparatus at 4°C. After marking the position of the lanes, the filter was immersed in 1× PBS containing 5% non-fat milk powder and 0.2% Tween 20. The filter was incubated with gentle shaking at room temperature for one hour and washed three times (10 min) with 1× PBS containing 0.05% Tween 20. The filter was then incubated with various antibodies. The antibodies used included an IgG_{2a} monoclonal antibody that recognizes an epitope near the C-terminal domain of p34^{cdc2} (Pharmingen) and a monoclonal antibody raised against a trp E-Rb fusion protein (PMG 245, gift from WenHwa Lee, University of Texas Medical Center at San Antonio). After incubation for 4 to 24 hours with the antibody, filters were washed three times (10 min) with 1× PBS containing 0.05% Tween 20. For studies utilizing pRB antibody, filters were then incubated for 30 minutes with a rabbit anti-mouse secondary antibody and washed three times as described above. The filters were then incubated (30 min) with a goat anti-rabbit horseradish peroxidase conjugated antibody, washed as described, and immersed for one minute in ECL luminol (Amersham). Filters were rapidly dried and exposed to Kodak XAR film for 30 to 90 seconds. When the antibody directed against p34^{cdc2} was used, after incubation with the antibody, the filter was washed as described then incubated with about 0.5 mCi ^{125}I-labeled protein A (Amersham) in 1x PBS containing 0.05% Tween for one hour. The filter was then washed as described and exposed to film overnight at −70°C between intensifying screens.

Protein assay

Protein was estimated by modified Bradford method (Bio-Rad) using bovine serum albumin as a standard.

Statistical analyses

All calculations and analyses were carried out using an ATT PC-6300 desktop computer and ABSTAT software. All data were expressed as the mean ± SEM. Statistical analyses were performed using paired and unpaired Student's t-test and ANOVA where appropriate. A P value of < 0.05 was considered significant.

Results

We first examined the effect of several putative growth factors on LLC-PK$_1$ [^{3}H]-thymidine uptake. The effects of 24 hours exposure of LLC-PK$_1$ cells to various concentrations of these growth factors are depicted in Figure 1. Nearly identical responses were observed when cells were given the growth factors with or without concomitant 6% fetal calf serum. In these studies, the phorbol ester phorbol 12-myristate 13-acetate (PMA) and TGF-α both stimulated [^{3}H]-thymidine uptake in a

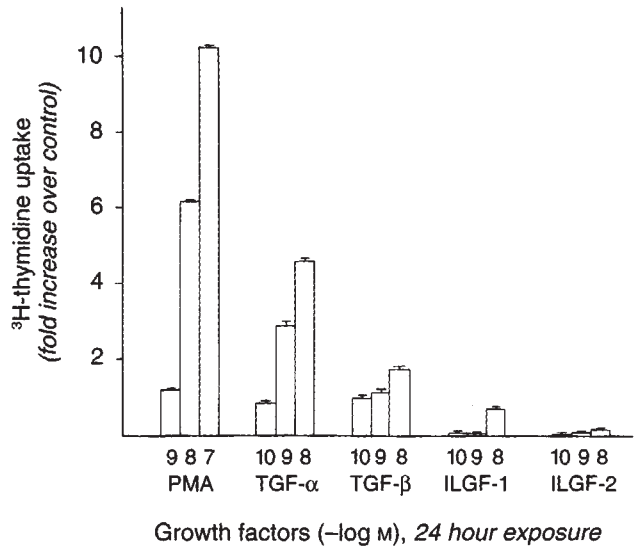

Fig. 1. *Effect of 24 hours exposure to several putative renal growth factors on LLC-PK$_1$ cell [^{3}H]-thymidine uptake.* The data represent the mean ± SEM of 4 to 6 experiments.

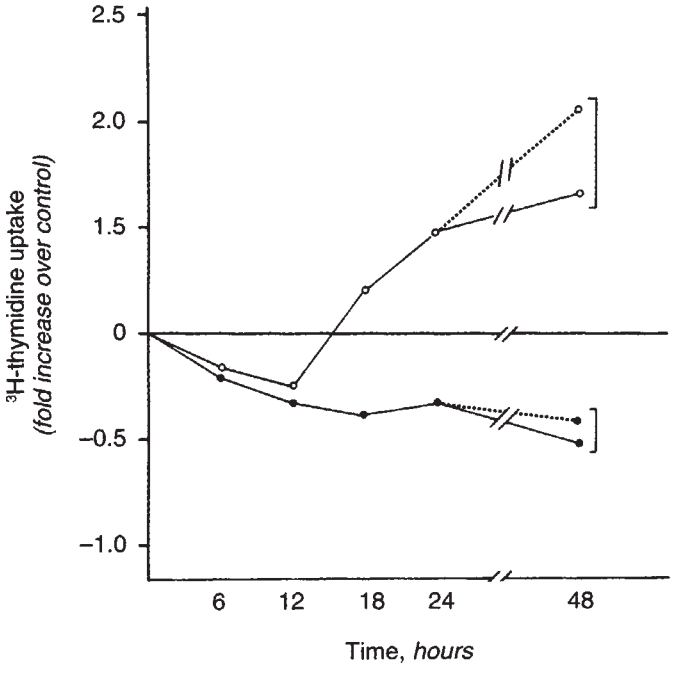

Fig. 2. *Time course of effect of TGF-β_1 (10^{-9} M) on [^{3}H]-thymidine uptake in LLC-PK$_1$ (○) and MDCK (●) cells.* Interrupted line is from 24 to 48 hours represent cultures that had TGF-β_1 removed. The data represent the mean values of 4 experiments. The SEM for each time point was less than 0.15-fold.

dose-dependent fashion. To our surprise, TGF-β_1 also modestly stimulated [^{3}H]-thymidine uptake while ILGF-1 and ILGF-2 exhibited limited ability to promote [^{3}H]-thymidine incorporation.

Proliferative responses to TGF-β_1 are exceptionally unusual in well-differentiated epithelial cells. Therefore, additional studies were undertaken. First, we examined the time course of 10^{-9} M TGF-β_1 on [^{3}H]-thymidine uptake in LLC-PK$_1$ cells (Fig. 2). A biphasic pattern was observed with inhibition of uptake observed after 6 and 12 hours exposure to TGF-β_1 followed by modest stimulation of uptake after 18, 24 and 48 hours exposure. Removal of TGF-β_1 from the media after 24 hours exposure was associated with a further modest increase in [^{3}H]-thymidine uptake when compared with cells that continued to be exposed to TGF-β_1. To determine if this pattern of response was unique for LLC-PK$_1$ cells, another renal epithelial cell line was studied (Fig. 2). In contrast to LLC-PK$_1$ cells, MDCK cells did not escape from the anti-proliferative effect of TGF-β_1 over 48 hours. The data depicted in Figure 2 were performed in cells that were maintained serum-free for 48 hours. At time 0, both 6% calf serum 10^{-9} M TGF-β_1 were added for the indicated time periods.

Next, the effect of exposure for 24 hours to 10^{-9} M of TGF-β_1, EGF and TGF-α when given alone and together on [^{3}H]-thymidine uptake was examined (Fig. 3). As expected, TGF-α and EGF modestly stimulated [^{3}H]-thymidine uptake and these effects were not additive. The effect of TGF-β_1 to stimulate [^{3}H]-thymidine uptake in LLC-PK$_1$ cells, however, appeared to be, at least in part, additive to the effects of TGF-α and EGF. By contrast, in MDCK cells, TGF-β_1 pre-treatment converted stimulatory effects of EGF and TGF-α on [^{3}H]-thymidine uptake into inhibitory effects as reported in other cell lines [19]. To determine if TGF-β_1 affects LLC-PK$_1$ cell number, a hemocytometer was used to count control cells and cells treated for 12 to 15 and for 24 to 72 hours with TGF-β_1. In these studies, counts of cells treated with TGF-β_1 for 12 to 15 hours averaged 79 ± 2% of control (untreated) values while counts of cells treated with TGF-β_1 for 24 to 72 hours were 125 ± 4% of control. Together, these observations suggest that LLC-PK$_1$ cells exhibit a unique response to TGF-β_1 treatment causing initial growth inhibition followed by stimulation of proliferation. Moreover, the response to TGF-β_1 to stimulate [^{3}H]-thymidine uptake in LLC-PK$_1$ cells appears, at least in part, additive to that of TGF-α and EGF.

The response of TGF-β_1 exposed LLC-PK$_1$ cells to escape from growth suppression and to proliferate in response to TGF-β_1 could be explained by production of an overriding growth factor. To study this possibility, the effects of media removed from LLC-PK$_1$ cells treated for 48 hours with 10^{-9} M TGF-β_1 and of media removed from control LLC-PK$_1$ cells not treated with TGF-β_1 but observed over an identical 48 hour period on [^{3}H]-thymidine uptake were compared. In these studies, after eight hours exposure to either the conditioned or the control media, [^{3}H]-thymidine was added to the media and the cells harvested four hours later. The uptake of [^{3}H]-thymidine in LLC-PK$_1$ cells exposed to the conditioned media was less than in cells exposed to control media (8,460 ± 285 vs. 10,371 ± 315 cpm, respectively, $N = 6$, $P < 0.05$). This 18% inhibition of [^{3}H]-thymidine uptake produced by conditioned media containing TGF-β_1 at 10^{-9} M was nearly identical to that observed (20 ± 3%) with LLC-PK$_1$ cells exposed to 10^{-9} M TGF-β_1 for 12 hours in non-conditioned media. In additional studies, conditional media was concentrated four- to sixfold. In these studies, [^{3}H]-thymidine uptake in LLC-PK$_1$ cells exposed to the concentrated, conditioned media containing TGF-β_1 exceeded that in cells exposed to concentrated, control media (13,609 ± 872 vs. 12,000 ± 386 cpm, respectively; $N = 8$, NS).

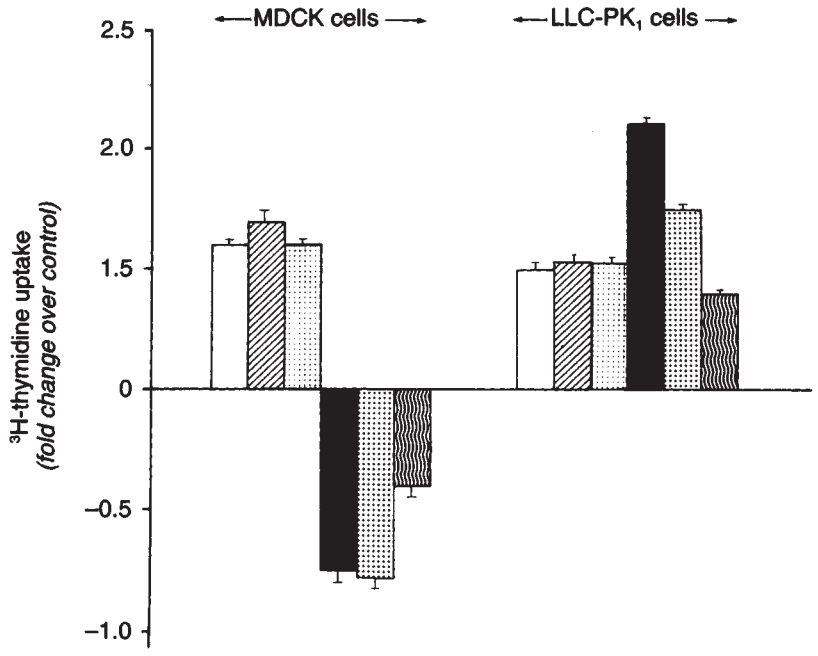

Fig. 3. *Effect of TGF-α (10^{-8} M, □), EGF (10^{-8} M, ▨) and TGF-β_1 (10^{-9} M, ▩) alone and together [(▦) TGF-α + EGF; (■) TGF-α + TGF-β; (⊡) EGF + TGF-β] on [^{3}H]-thymidine uptake in MDCK cells (6 bars on left) and LLC-PK$_1$ cells (6 bars on right).* The values represent the mean ± SEM of 5 experiments. The noted growth factors and 6% calf serum were added simultaneously to cells that were serum free for 48 hours prior to study.

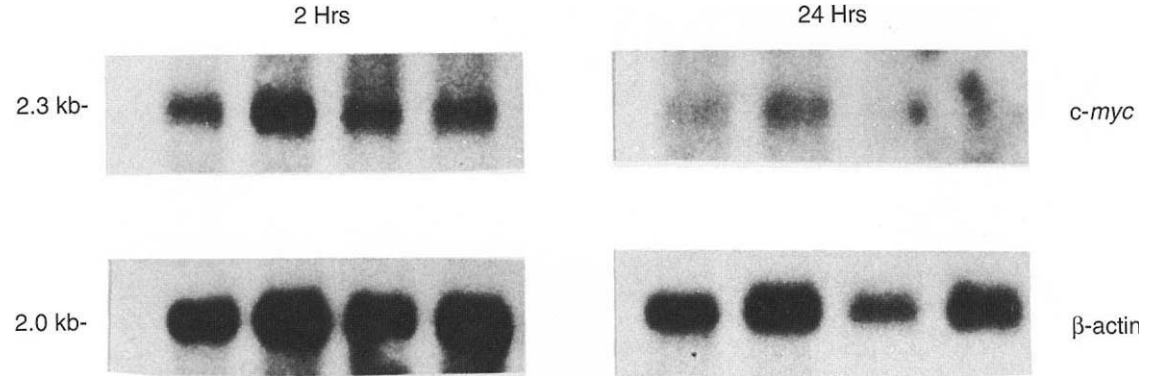

Fig. 4. *Northern blots of* c-myc *(upper panels) and β-actin (lower panels) mRNA after 2 hours (left four panels) and 24 hours (right four panels) exposure to TGF-β_1 in LLC-PK$_1$ cells.* First lane of each panel represents control, second lane 6 hours exposure to EGF (10^{-8} M), third lane TGF-β_1 (10^{-9} M) and fourth lane TGF-β_1 (10^{-9} M) plus EGF (10^{-8} M). All studies were performed concurrently.

Studies in multiple cell types suggest that the anti-proliferative effect of TGF-β_1 is associated with decreased expression of the c-*myc* proto-oncogene [5–15]. We therefore studied Northern blots for c-*myc* mRNA isolated from LLC-PK$_1$ and MDCK cells exposed to EGF in the presence and absence of pretreatment with TGF-β_1. The effect of short-term (6 hr) exposure to 10^{-9} M TGF-β_1 on 10^{-8} M EGF-stimulated c-*myc* expression was first examined (Fig. 4). When the ratio of c-*myc* to β-actin signal was meaned for five experiments, TGF-β_1 modestly decreased EGF-stimulated c-*myc* expression in both LLC-PK$_1$ (40 ± 8%) and MDCK (35 ± 5%) cells. Next, the effect of longer (18 to 24 hr) exposure to TGF-β_1 was examined (Fig. 5). In the experiments (N = 5), TGF-β_1 decreased the effect of exposure to EGF to stimulate c-*myc* expression by 30 ± 8% in LLC-PK$_1$ cells and by 38 ± 7% in MDCK cells. These results demonstrate that TGF-β_1 comparably inhibits EGF-stimulated c-*myc* expression in cells that escape (LLC-PK$_1$) and do not escape (MDCK) from TGF-β_1 inhibition of proliferation as well as at time points when TGF-β_1 treated LLC-PK$_1$ cells exhibit inhibition (6 hr) as well as stimulation (18 to 24 hr) of [^{3}H]-thymidine uptake.

We next undertook preliminary studies to clarify possible mechanism whereby brief (less than 12 hr) exposure to TGF-β_1 inhibits LLC-PK$_1$ proliferation. Since the product of the retinoblastoma tumor suppressor gene has been implicated in regulation of TGF-β_1 inhibition of proliferation and suppression of c-*myc* transcription [10–13, 16], we probed whole cell lysates of LLC-PK$_1$ cells with an antibody directed against pRB. The results (Fig. 6) suggest that TGF-β_1 pretreatment may decrease steady-state expression of pRB. However, exposure to EGF appears to increase pRB expression in both the absence of presence of TGF-β_1. We also utilized Western blots of whole cell lysates to examine if the expression of p34^{cdc2}, a cell cycle protein that may be involved in proliferation, is affected by

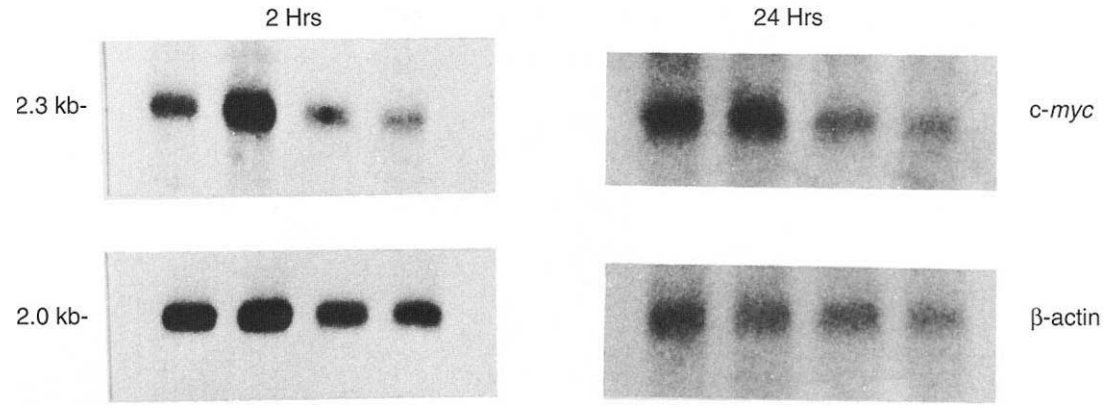

Fig. 5. *Northern blots of* c-myc *(upper panels) and β-actin (lower panels) mRNA after 2 hours (left panels) and 24 hours (right panels) exposure to TGF-β_1 in MDCK cells.* First lane of each panel represents control, second lane 6 hours exposure to EGF (10^{-8} M), third lane TGF-β_1 (10^{-9} M) and fourth lane TGF-β_1 (10^{-9} M) plus EGF (10^{-8} M).

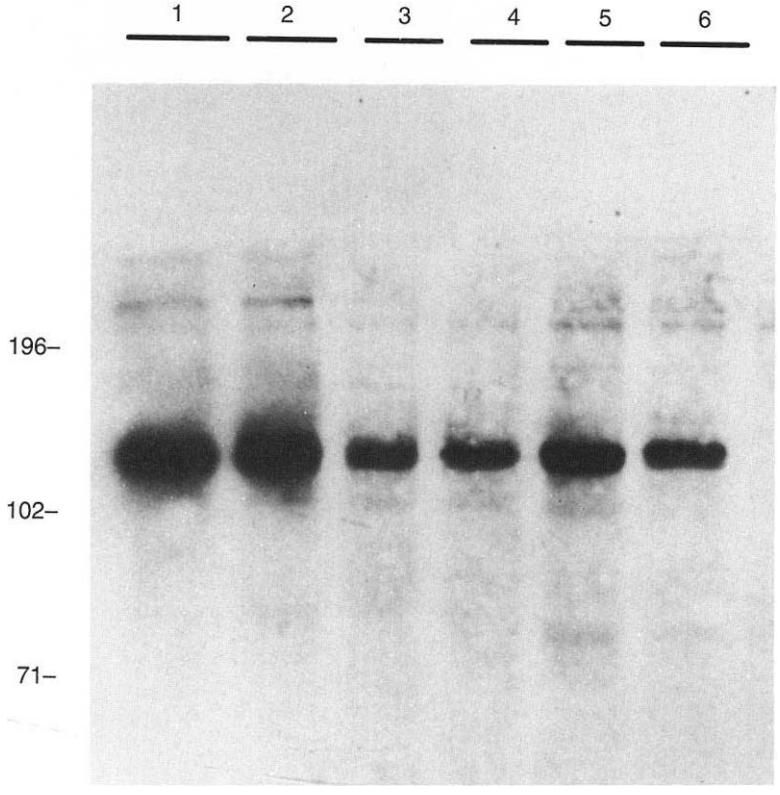

Fig. 6. *Western blot of LLC-PK$_1$ cell lysates probed with an antibody directed against retinoblastoma protein.* The first lane is control, second lane 2 hours exposure to EGF (10^{-8} M), third lane 6 hours exposure to EGF, fourth lane TGF-β_1 (10^{-9} M) for 2 hours preceding 2 hours exposure to EGF (10^{-8} M) and sixth lane TGF-β_1 (10^{-9} M) for 2 hours preceding 6 hours exposure to EGF (10^{-8} M).

EGF and TGF-β_1. Western blots probed with an antibody directed against a carboxy terminal epitope of $p34^{cdc2}$ did not demonstrate a major effect of TGF-β_1 to alter EGF-stimulated steady state expression of $p34^{cdc2}$ (Fig. 7).

Discussion

TGF-β_1 is of interest with regard to the kidney because it has been shown to be an important regulator of renal tubular and glomerular mesangial and epithelial cell proliferation [19–22]. Moreover, TGF-β_1 may regulate renal epithelial cell production of extracellular matrix, facilitate intercellular communication and modulate cell phenotype [19, 23, 24]. Transforming growth factor β_1 also may be a pathogenetic regulator in renal response to a variety of insults including glomerulonephritis [25, 26], obstructive uropathy [27] and diabetes mellitus [28, 29]. The present studies were therefore undertaken to gain a better understanding of the mechanisms whereby TGF-β_1 regulates renal tubular epithelial cell proliferation.

The major finding of the present study is that a well-differentiated pig kidney epithelial cell line (LLC-PK$_1$ cells) exhibits unique responses to TGF-β_1. Initially, TGF-β_1 inhibits [^{3}H]-thymidine uptake and proliferation. This inhibition lasts for

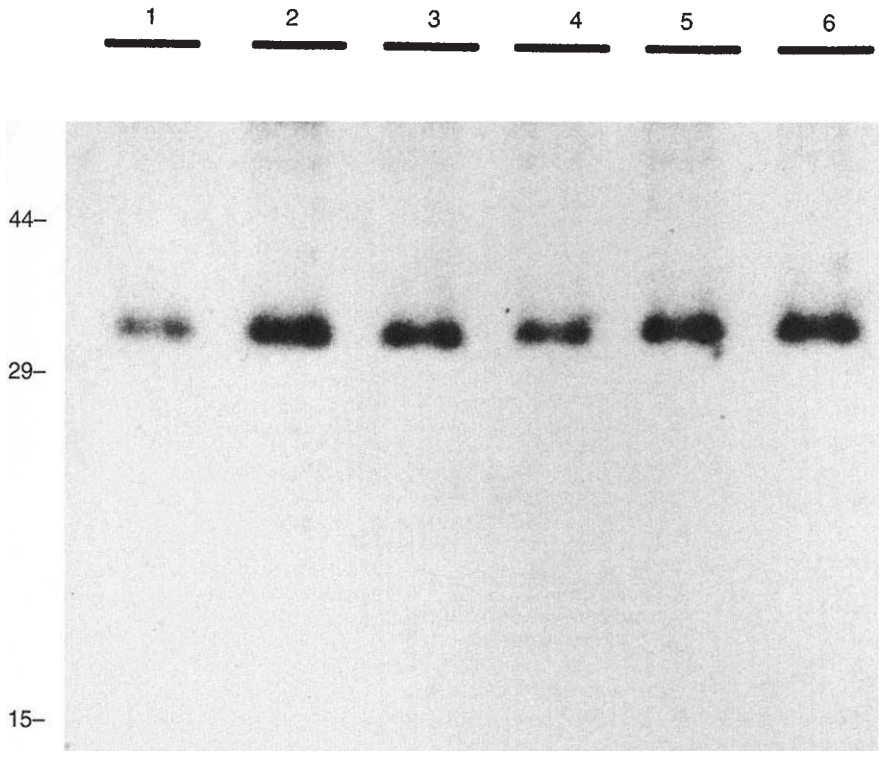

Fig. 7. *Western blot of LLC-PK$_1$ lysates probed with an antibody directed against a portion of the p34^{cd2} protein.* The conditions of lanes 1 through 6 are identical to the conditions for lanes 1 through 6 described in the legend to Figure 6.

about 12 to 18 hours and is followed by stimulation of [^{3}H]-thymidine uptake and proliferation. The stimulation of [^{3}H]-thymidine uptake following 24 hours exposure to TGF-β_1 is, at least in part, additive to that produced by TGF-α and EGF. This biphasic response of LLC-PK$_1$ cells to TGF-β_1 is not a special characteristic of renal tubular epithelial cells since TGF-β_1 persistently inhibits basal, TGF-α- and EGF-stimulated [^{3}H]-thymidine uptake in another renal tubular epithelial cell line (MDCK cells). TGF-β inhibits [^{3}H]-thymidine uptake in the vast majority of well-differentiated epithelial cells in which it has been studied [1–17, 19]. Resistance to TGF-β-induced proliferation has been reported to occur very rarely (<0.01%) in well-differentiated human colon cancer cells and only transiently in Mv1Lu mink lung epithelial cells despite exposure to TGF-β_1 for several days [8, 29]. Resistance to TGF-β inhibition of cell growth is more commonly observed in human cancer cells including those of retinoblastoma, colon, breast, cervical, bladder and skin origin [29–35]. Also, chemical mutagenesis as well as introduction of the E1A and v-*raf* oncogenes can result in resistance to TGF-β inhibition of growth [35, 36]. Although these transformed cells can develop resistance to TGF-β_1, a proliferative response as observed in the present studies has been found only in fibroblasts grown in an anchorage independent mode [4].

Two potential explanations for the development of resistance to TGF-β_1 growth inhibition include either neutralization of TGF-β or production of an overriding growth factor. With regard to possible neutralization of TGF-β_1, in some cell types TGF-β_1 induces production of the proteoglycan decorin which binds TGF-β_1 thereby preventing access to its receptor [39]. With regard to an overriding growth factor, in mesangial cells TGF-β_1 can induce genes for the platelet-derived growth factor [20]. The observations of the present study that concentrated conditioned media from TGF-β exposed cells no longer inhibit [^{3}H]-thymidine uptake may support the presence of either an overriding growth factor or a TGF-β inhibitor. However, our conditioned media experiments are not definitive and further studies are required to resolve this issue.

It is also possible that TGF-β resistance could be explained by a change in TGF-β receptor type and/or characteristics. For example, malignant and chemically mutagenized cells that are resistant to TGF-β_1 lose TGF-β_1 binding ability [34, 36, 40]. In those cell lines that are resistant to TGF-β_1, restoration of either both type I and type II or only type II TGF-β receptors by hybridization techniques restores TGF-β_1 inhibition of proliferation [29, 34, 40]. However, loss of TGF-β_1 receptors does not readily explain the TGF-β_1 enhancement of [^{3}H]-thymidine uptake that we observed. We also found that at a time at which LLC-PK$_1$ cells have escaped from the anti-proliferative effect of TGF-β_1, TGF-β_1 treated cells continue to exhibit suppression of EGF stimulated c-*myc* expression suggesting the presence of a functional TGF-β_1 receptor linked to one of the known TGF-β post-receptor signaling pathways. Although studies of TGF-β_1 receptors are clearly needed to clarify our observations, some of our results suggest that post-receptor events contribute, at least in part, to the TGF-β resistance that we observed.

The post-receptor signaling pathways involved in TGF-β_1 biological effects have not been totally clarified. Our previous investigations suggest that cyclic AMP and protein kinase A may be involved [18]. In other studies, a consistent observation has been that TGF-β_1 inhibition of proliferation occurs in parallel with suppression of basal and growth factor-stimulated transcription of the c-*myc* oncogene, an oncogene which is

associated with cell proliferation [8–15]. For example, cells that are resistant to the growth inhibitory properties of TGF-β_1 do not exhibit decreased c-*myc* expression following TGF-β_1 [8, 30]. The precise mechanism(s) whereby TGF-β_1 leads to suppression of c-*myc* expression remains unclear. Most [10–12] but not all studies [13] suggest that the product of the retinoblastoma tumor suppressor gene (pRB) plays a critical role in mediating TGF-β suppression of c-*myc* transcription. We therefore examined EGF-stimulated c-*myc* expression in the present studies. Our results demonstrate that escape from TGF-β_1 growth inhibition in LLC-PK$_1$ cells occurs despite continued modest suppression of c-*myc* expression as determined by Northern analyses. Further studies are needed to determine the mechanism of this decrease in c-*myc* expression. We also used Western blots to examine the short-term effects of EGF and TGF-β_1 (alone and together) on LLC-PK$_1$ expression of pRB. These results suggest that EGF may increase and TGF-β_1 decrease steady state expression of pRB. Clearly, more studies are also needed to clarify the relationship between TFG-β_1, EGF and pRB in LLC-PK$_1$ cells.

Recently, a post-translational relationship between pRB and c-*myc* protein has also been found [41, 42]. These two proteins can physically bind to each other [32] and microinjection studies suggest that these proteins antagonize the growth effects of one another within the cell [42]. These observations are of particular interest with regard to TGF-β_1 resistance in view of the recent findings of Missero and colleagues [37, 38]. In these studies, TGF-β_1 resistance occurs in keratinocytes transformed with the E1A oncogene. This resistance correlates closely with the ability of EIA proteins to bind pRB and three other cellular proteins including a cdc3-associated cell-cycle regulatory protein [37]. Binding of E1A to subsets of these four proteins induced only partial resistance to TGF-β_1 [37]. More recently, Koff and coworkers found that TGF-β inhibition of proliferation in Mv1Lu cells was associated with failure to assemble functional cyclin and cyclin dependent kinases and these effects correlated with decreased pRB phosphorylation [17]. In the present studies, we did not find an effect of either EGF TGF-β_1 to regulate steady-state expression of a p34^{cdc} kinase. It is clear that further studies examining the role of pRB, cyclin and cyclin-dependent kinases are required to clarify the mechanisms of the escape from TGF-β_1 inhibition of growth that we observed in LLC-PK$_1$ cells.

In summary, the present results find that LLC-PK$_1$ renal epithelial cells develop rapid, *in vitro* resistance to the growth inhibitory properties of TGF-β_1. This unique response to TGF-β_1 in LLC-PK$_1$ renal tubular epithelial cells is not seen in another renal epithelial cell line (MDCK cells). The proliferative response to TGF-β_1 in LLC-PK$_1$ cells is additive to that of other growth factors and occurs despite the continued effect of TGF-β_1 to suppress EGF-stimulated c-*myc* expression. The ability of LLC-PK$_1$ renal epithelial cells to escape from TGF-β growth inhibition provides an excellent opportunity to delineate the mechanism(s) whereby TGF-β and other growth factors exert their effects on renal tubular epithelial cell growth. In particular, further studies to delineate the possible role of reduced pRB expression and other cellular factors in these unique LLC-PK$_1$ responses to TGF-β_1 will be of great interest.

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Reprint requests to Robert J. Anderson, M.D., Medical Service (111), Denver Veterans Affairs Medical Center, 1055 Clermont Street, Denver, Colorado 80220, USA.

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